52

Guidance for Industry

ASSESSMENT OF THE EFFECTS OF ANTIMICROBIAL DRUG RESIDUES FROM FOOD OF ANIMAL ORIGIN ON THE HUMAN INTESTINAL FLORA

Final Guidance

This guidance discusses a recommended pathway approach for assessing the effects of antimicrobial drug residues in food on the human intestinal flora. This document supercedes the current guidance #52, Guideline for Microbiological Testing of Antimicrobial Drug Residues in Food, published in January of 1996.

Comments and suggestions regarding this document should be sent to the Division of Dockets Management (HFA-305), Food and Drug Administration, 5630 Fishers Lane, Room 1061, Rockville, MD 20852. All coinments should be identified with the Docket No. 93D-0398. Submit electronic comments at http://www.fda.gov/dockets/ecomments.

For questions regarding this document, contact Haydée Fernández, Division of Human Food Safety (HFV-153), Center for Veterinary Medicine, Food and Drug Administration, 7500 Standish Place, Rockville, MD 20855, 301-827-6981, E-mail: afernand@cvm.fda.gov.

Additional copies of this final guidance may be requested from tlie Cominunications Staff (HFV-12), Center for Veterinary Medicine, Food and Drug Administration, 7519 Standish Place, Rockville, MD 20855 and may be viewed on the Internet at http://www.fda.gov/cvm.

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U.S. Department of Health and Human Services
Food and Drug Administration
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ASSESSMENT OF THE EFFECTS OF ANTIMICROBIAL DRUG RESIDUES FROM FOOD OF ANIMAL ORIGIN ON THE HUMAN INTESTINAL FLORA[1]

This guidance represents the agency's current thinking on the approach that should be used to assess the microbiological safety of antimicrobial drug residues in food of animal origin. It does not create or confer any rights for or on any person and does not operate to bind FDA or the public. You can use an alternative approach if the approach satisfies the requirements of the applicable statutes and regulations. If you want to discuss an alternative approach, contact the **FDA** staff responsible for implementing this guidance. If you cannot identify the appropriate FDA staff, call the appropriate number listed on the title page of this guidance.

I. INTRODUCTION

The human intestinal flora is a balanced ecosystem that is very important in maintaining an individual's health. Although this system is generally stable, clinical studies have shown that therapeutic doses of antimicrobials may change the balance. (1,2,3) The type or extent of change in the system will depend on the spectrum of action of the antimicrobial drug, its dose, and the length of an individual's exposure to the drug. The lowest concentration of any antimicrobial drug that can affect the intestinal flora is not clear. However, studies in ***in vitro*** (continuous or semi-continuous flow culture systems) and ***in vivo*** human flora-associated rodent (rodents implanted with human fecal flora) test systems and in human volunteers have shown that low levels of antimicrobial drugs are capable of altering different parameters of the intestinal flora depending on the spectrum of action and concentration of drug. (4, 5, 2, 3)

The main adverse effects of antimicrobial drugs on the human intestinal flora are selection of resistant bacteria and disruption of the colonization barrier (or barrier effect) of the resident intestinal flora. Colonization barrier or barrier effect is the "limiting action" of the normal flora on colonization of the bowel by exogenous or indigenous potentially pathogenic microorganisms. (6) Other effects, such as alteration of the metabolic activity of the flora, may be important, also.

Regulators and sponsors of new animal drugs have an interest in establishing relevant and validated methods for determining the effects of microbiologically active animal drug residues on the human intestinal flora. Any such effects should be assessed in the human food safety evaluation of new animal drugs intended for use in food-producing animals. Among the ***in vitro*** and ***in vivo*** approaches currently used to study the effect of antimicrobial drugs on the **human** intestinal flora are quantitative ***in vitro*** antimicrobial drug susceptibility testing, static batch cultures, semi-continuous **and** continuous flow

[1] This guidance has been prepared **by** the Office of New Animal Drug Evaluation in the Center for Veterinary Medicine at the Food and Drug Administration.

culture systems, simulated gut models, human volunteers, conventional animals, gnotobiotic rodents, and human flora-associated rodents.

CVM has decided to modify Guidance No.52 "Microbiological Testing of Antimicrobial Drug Residues in Food" published in January of 1996[7] based on information made available after that time concerning the effects of low doses of different classes of antimicrobial drugs on the human intestinal flora. Therefore, CVM is recommending that sponsors use a "pathway approach" (described below) for addressing the human food safety of antimicrobial drug residues rather than the approach described in the 1996 version of the guidance. The scientific rationale for this decision and the FDA/CVM regulatory history on this issue are provided in a separate document available to the public at the Division of Dockets Management (HFA-305), Food and Drug Administration, 5630 Fishers Lane, rm. 1061, Rockville, MD 20852. ("History and Scientific Issues Related to Guidance #52")

The pathway approach presented in this guidance document represents a general approach for assessing the microbiological safety of antimicrobial drug residues in food. If further microbiological studies are warranted for determining the Acceptable Daily Intake (ADI) for a new animal antimicrobial drug, the sponsor of that drug is encouraged to contact the Center to discuss the appropriate test systems and protocols for the studies.

CVM is aware that the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) is currently drafting a related guideline and that this guidance may be superceded at a later date by a guidance document published by the VICH.

FDA's guidance documents, including this guidance, do not establish legally enforceable responsibilities. Instead, guidances describe the Agency's current thinking on a topic and should be viewed only as recommendations, unless specific regulatory or statutory requirements are cited. The use of the word "should" in Agency guidances means that something is suggested or recommended, but not required.

II. PATHWAY APPROACH FOR ADDRESSING THE EFFECTS OF ANTIMICROBIAL DRUG RESIDUES FROM FOOD OF ANIMAL ORIGIN ON THE HUMAN INTESTINAL FLORA

The conditions and rationale for addressing the microbiological safety of antimicrobial drug residues in food are simplified in a chart at the end of this section.

The microbiological safety of antimicrobial drug residues in food is an important issue that should be addressed by the sponsor of a new animal drug. An assessment of the safety of antimicrobial drug residues in food should be part of the human food safety component of new animal drug applications for antimicrobial drugs. If these residues have no antimicrobial activity against representatives of the human intestinal flora *(E.*

coli, and species of *Bacteroides, Bifidobacterium, Clostridium, Enterococcus, Eubacterium (Collinsella), Fusobacterium, Lactobacillus, Peptostreptococcus/Peptococcus*), an ADI should be calculated based on traditional toxicology studies. However, if the residues have antimicrobial activity, the sponsor should address the potential availability of these microbiologically active residues in the human colon. It should be assumed that the human colon would be exposed to all residues present in the edible tissues, unless the sponsor can demonstrate through reference to controlled experimentation in humans or animals (e.g., pharmacokinetic studies, bioavailability studies, etc.) that some or all of the residues have no potential to enter the colon.

If it is determined that microbiologically active residues can enter the colon, the sponsor should assess the potential of these residues to select for resistant bacteria, disrupt the protective barrier effect provided by the intestinal flora, or otherwise alter the balance of intestinal flora. The sponsor may attempt to demonstrate that the residues are metabolized rapidly to microbiologically inactive compounds or are rapidly bound to intestinal contents and rendered microbiologically unavailable in the human colon. Alternatively, if the antimicrobial residues are not metabolized or bound such that they are microbiologically inactive, the sponsor should determine if these residues would cause adverse effects on the intestinal flora. This could be done using appropriate human data, if available, or data from *in vitro* or *in vivo* test systems. The adverse effects of human health concern that should be considered are the potential of these residues to select for resistant bacteria, disrupt the protective barrier effect provided by the intestinal flora, or otherwise alter the balance of intestinal flora. If no information is available, the sponsor should perform studies using an *in vitro* or **an** *in vivo* test system to determine the endpoint(s) of human health concern.

The sponsor may wish first to perform preliminary studies such as batch cultures with fecal suspensions or an *in vivo* preliminary study to determine which microbiological endpoint is suspected to be altered by the drug. The sponsor may also choose to perform a definitive study using an *in vitro* or an *in vivo* test system to study the effect of the drug on the endpoints of human health concern and determine the no-observable effect concentrations/no-observable effect levels (NOECs/NOELs) for these endpoints. However, if the endpoint(s) of concern have been determined, definitive studies using *in vitro* or *in vivo* test systems should be performed to determine the NOEC/NOEL for the drug on the chosen endpoint(s).

If disruption of the colonization barrier is the endpoint of concern, either *in vitro* (e.g., continuous or semi-continuous culture systems) or *in vivo* test systems (e.g., human flora-associated rodent test systems) are preferable for determining a NOEL for this endpoint, as opposed to *in vitro* antimicrobial susceptibility testing to generate minimum inhibitory concentration (MIC) data. This is because these systems have the potential to better approximate the effects of microbial interactions and high bacterial densities than MIC data. However, the sponsor could use MIC data as an option to estimate a conservative microbiological ADI. MIC data indirectly assess changes in bacterial populations. If the antibiotic concentration is below the levels that inhibit cell growth, it could be assumed

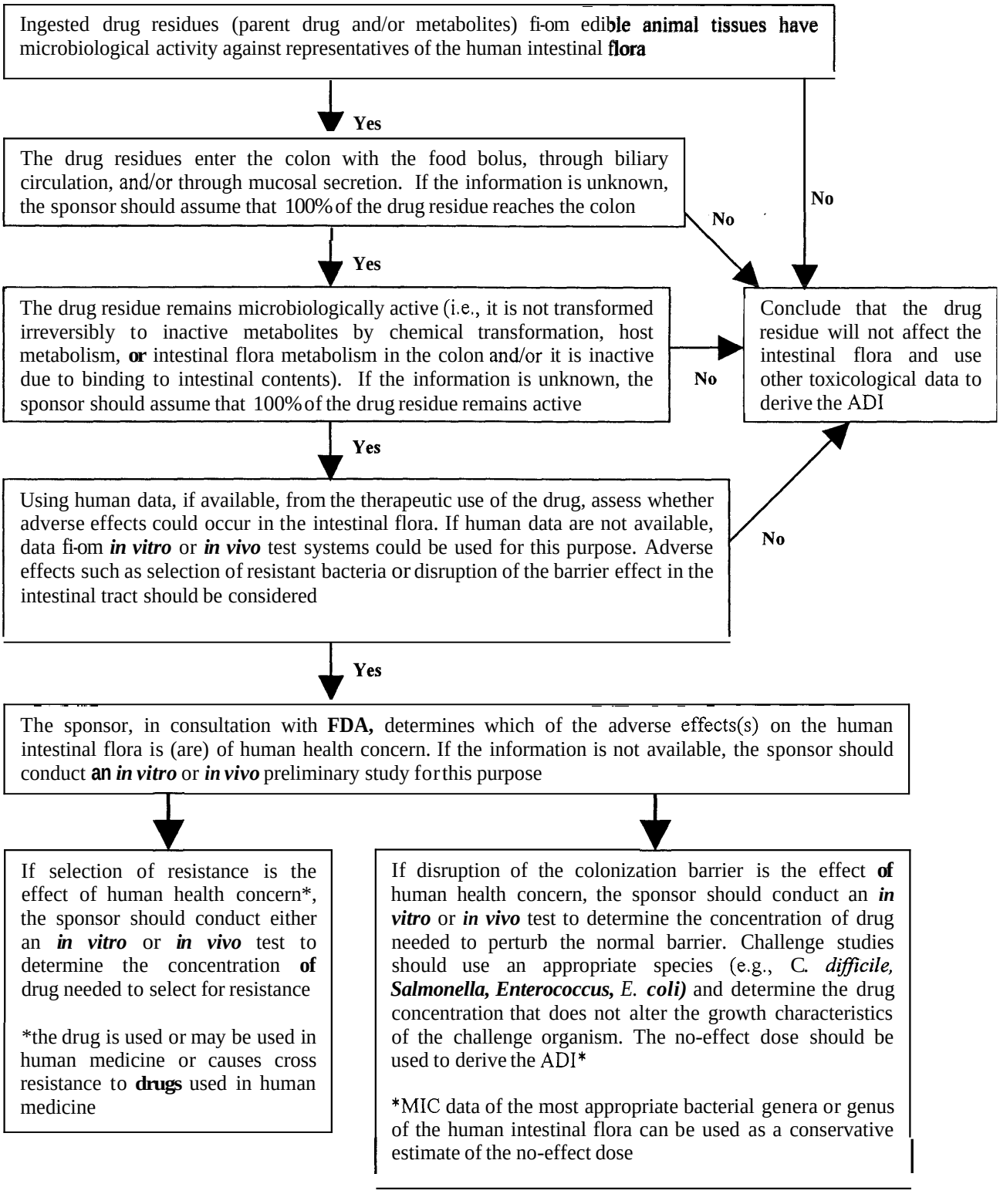
that the bacteria responsible for the barrier effect would not be affected. Vice versa, if bacteria are not allowed to grow due to low concentrations of antimicrobials, an unbalanced flora could allow the establishment of a pathogen that is not sensitive to the antibiotic in study.

The ADI derived from MIC data is conservative because the inoculum density used for testing is orders of magnitude lower than the bacterial population of the colon. In addition, the growth conditions in MIC testing (growth medium, pH, lack of fecal solids, lack of microbial interactions and drug metabolism, etc.) minimize the potential of drug inactivation. If MIC testing is used to derive an ADI, the median MIC obtained by standard methods such as those of the National Committee for Clinical Laboratory Standards (NCCLS) should be used to determine a NOEC. It is recommended that at least 10 isolates from each of the most representative bacteria listed on pages 4 and 5 of this guidance be obtained from healthy human volunteers.

Although the use of MIC data may be an option to derive an ADI, CVM does not encourage the use of this data for determining the NOEC for disruption of the colonization barrier of the human intestinal flora. Quantitative *in vitro* determinations of antimicrobial susceptibility do not reflect or account for factors such as bacterial population density, pH, intestinal growth conditions, bacterial metabolism, bacterial antagonism, or other factors of relevance to the human colonic flora. A more appropriate NOEC may be obtained through other test systems that better model the intestinal flora.

If the endpoint of concern is the selection of resistant bacterial strains, the sponsor should conduct *in vitro* or *in vivo* studies in test systems to determine a NOEL for this endpoint. Quantitative *in vitro* determinations of antimicrobial susceptibility, leading to the generation of MIC data that is coupled to the effects generated in the test system(s), should be an element of this analysis.

PATHWAY APPROACH FOR ADDRESSING THE EFFECTS OF ANTIMICROBIAL DRUG RESIDUES FROM FOOD OF ANIMAL ORIGIN ON THE HUMAN INTESTINAL FLORA



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History and Scientific Issues Related to Guidance #52

HISTORY

In the Federal Register of January 30, 1996 (61 FR 3043), the Center for Veterinary Medicine (CVM, the Center) published a Notice of Availability of Guidance Document No. 52 "Microbiological Testing of Antimicrobial Drug Residues in Food" (Guidance No. 52). This document stated that the CVM considers antimicrobial activity as a valid endpoint for establishing tolerances for antimicrobial drugs. The guidance also stated that antimicrobial drug residues present in food of animal origin should not cause any adverse effects on the ecology of the human intestinal flora of consumers. The guidance identified antimicrobial drugs that would be exempt from additional microbiological testing and those that would warrant testing. The reasons for exempting certain antimicrobial drugs from additional microbiological testing included "very low" residues present in the food, residues with limited antimicrobial activity, and antimicrobial drugs with no adverse effects on the human intestinal flora at doses approved for the target species (1).

Guidance No. 52 stated that "very low" levels of antimicrobial drug residues present in food of animal origin do not disrupt the intestinal flora or select for resistant microorganisms and, therefore, would be "safe" under Section 512 of the Federal Food, Drug, and Cosmetic Act (the Act). Based on the best information available at that time, the CVM believed that a maximum Acceptable Daily Intake (ADI) of 1.5 mg/person/day of microbiologically active antimicrobial drug residues present in the food qualified as "very low" residues and should not produce adverse effects on the intestinal flora (1). After CVM established the maximum ADI of 1.5 mg/person/day in the 1996 version of Guidance 52, CVM staff publicly stated (e.g., at a workshop sponsored by FDA on September 20 and 21, 1999, in Rockville, Maryland) that this threshold would need to be re-evaluated when additional information was obtained on the adequacy of this number for different classes of antimicrobial drugs (2).

The 1996 guidance recommended that additional microbiological testing be performed for those antimicrobial drugs for which sponsors were seeking an ADI higher than 1.5 mg/person/day. The guidance document identified areas for which antimicrobial residues present a potential public health concern. The endpoints addressing the areas of concern are: 1) changes in the metabolic activity of the intestinal flora; 2) changes in antimicrobial susceptibility patterns of the intestinal flora; 3) changes in the colonization resistance properties (barrier effect) of the flora; and 4) changes in the numbers and relative proportions of different bacterial species. The guidance recommended that sponsors characterize the product, identify its microbiological activity, and monitor the appropriate microbiological endpoints to establish the antimicrobial no-observed effect level (NOEL). Because no validated model systems were available at that time, the CVM announced its intention to validate model systems to evaluate the effect of low levels of antimicrobial drugs on endpoints of potential public health concern. The guidance also stated that *in vitro* minimum inhibitory concentration (MIC) data should not be submitted

to establish the microbiological NOEL because these data are not predictive of the concentrations of drug residues that are of potential public health concern. Sponsors were encouraged to consult with the CVM to determine appropriate protocols before conducting studies (1).

In 1995, the CVM funded two extramural research contracts to study the dose-response effects of antimicrobial drugs on human intestinal flora endpoints that could be of public health concern. **A** continuous flow one-chambered culture system inoculated with human intestinal flora and a human flora-associated (HFA) mouse model were studied as possible test systems for studying dose-response effects of low doses of antimicrobial drugs.

In a workshop entitled "Microbiological Safety of Antimicrobial Residues in Food" sponsored by **FDA** on September 20 and 21 **,** 1999, in Rockville, Maryland, information from the two FDA-funded research contracts was presented. Data on the effect of low doses of different classes of antimicrobial drugs on several microbiological endpoints of the human intestinal flora were discussed. After reviewing and discussing the data, the CVM concluded that the threshold ADI established in the 1996 version of Guidance No. 52 is not appropriate for all classes of antimicrobial drugs. Different classes of antimicrobial drugs affect to different degrees the microbiological endpoints that could be of public health concern. Consequently, the Center proposed at the workshop to replace the maximum ADI approach with a pathway approach for addressing the microbiological safety of antimicrobial drug residues in food (2).

On March 3, 2000, CVM published an update at the CVM homepage (CVM Updates) entitled "CVM Plans to Modify Guidance on Microbiological Safety of Drug Residues in Food" (3). This update stated that Guidance #52 would be modified and a notice of its availability would be published in the Federal Register, asking for comments. A notice announcing the availability of the draft modified Guidance #52 published on December 27, 2001 (4), and interested persons were given until March 27, 2002, to comment. CVM considered comments to the proposed guidance and published the availability of the final Guidance #52 entitled "Assessment of the Effects of Antimicrobial Drug Residues from Food of Animal Origin on the Human Intestinal Flora" in the Federal Register. CVM is aware that the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products **(VICH)** is currently drafting a related guideline and that this guidance may be superceded at a later date by a guidance document published by the VICH.

SCIENTIFIC ISSUES

A. Antimicrobials and the Human Intestinal Flora

The human intestinal flora, an ecologically balanced system that plays an important role in maintaining and protecting the health of individuals, is generally very difficult to alter. For example, diet does not significantly alter the ecology of the human intestinal flora. However, antimicrobial drugs may have a major effect on the ecology of the intestinal flora (5, 6, 7).

Studies demonstrate that therapeutic oral doses of antimicrobials that are poorly or incompletely absorbed, excreted in the bile, or reach the intestinal lumen through circulation and excretion from the intestinal mucosa can potentially alter the intestinal flora (2, 6, 8, 9). Some of these alterations may involve suppression of important bacteria and overgrowth of potentially pathogenic microorganisms that may cause systemic infections, especially in immunocompromised patients. **A** well-known example of overgrowth of potentially pathogenic bacteria is the infection caused by ***Clostridium difficile***. This anaerobic bacterium, that may be indigenous or acquired in the hospital environment, may overgrow after being subjected to antibiotic treatment, disrupt the intestinal flora, and produce toxins. ***Clostridium difficile*** toxins may damage the intestinal mucosa resulting in diarrhea that may range from mild to life-threatening pseudomembranous colitis **(6).**

Other effects of antimicrobial drugs include the selection of drug-resistant intestinal bacteria, the decrease of the colonization resistance properties of the flora (or barrier effect), and alteration of the metabolic activity of the intestinal bacteria (e.g., metabolism of neutral steroids and bile acids, conversion of bilirubin to urobilinogen, metabolism of drugs and other substances, synthesis of vitamins) (6, 9, 10, 11).

Different classes of antimicrobials may alter the intestinal flora differently. For example, most quinolone drugs suppress or sometimes eliminate Gram-negative bacteria (especially ***Enterobacteriaceae),*** partially affect some Gram-positive aerobic cocci, and have very little effect on anaerobic bacteria. These alterations are concentration- and drug-dependent **(9).**

Volunteers treated orally with **500** mg **of** ciprofloxacin every 12 hours for 7 days showed complete elimination of the coliform bacteria and a decrease in the number of streptococci and staphylococci by day 7; however, these counts returned to normal by day 14 (7 days after cessation of treatment). The anaerobic bacteria and yeasts were not affected and the flora returned to normal after cessation of ciprofloxacin treatment (12). Longer treatments with ciprofloxacin (500 mg given twice a day for 42 days to patients with leukemia) have shown to select for ciprofloxacin-resistant ***Pseudomonas*** and ***Acinetobacter*** **(13).**

Ciprofloxacin-resistant ***Escherichia coli*** has emerged in cirrhotic patients treated with 1000 mg **of** ciprofloxacin once a week for 12 weeks. Similar effects are seen with other quinolones (14). Sitafloxacin, a broad-spectrum fluoroquinolone with activity against

aerobic and anaerobic bacteria reduced the number of ***Bacteroides*** strains and other anaerobes; drastically reduced the number of ***Enterobacteriaceae,*** lactobacilli and bacilli; decreased the enterococci population in half of the volunteers; and increased the proportion of resistant ***Bacteroides*** strains to 6.25 μg/ml of drug in most patients treated orally with 100 mg of the drug three times a day for one week. Signs of recovery of the flora were seen on day **14** after cessation of treatment, but the recovery was still incomplete (e.g., the proportion of resistant ***Bacteroides*** increased during the treatment period but decreased after the treatment) (15).

Other classes of antibiotics such as beta-lactams, cephalosporins, tetracyclines, lincosamides and macrolides, aminoglycosides, etc. may also produce effects on the intestinal flora at therapeutic doses. Broad-spectrum penicillins at therapeutic doses have been shown to suppress the growth of aerobic Gram-negative bacteria and anaerobic flora, and promote the overgrowth of aerobic Gram-positive bacteria. **Also,** most cephalosporins induce overgrowth or new colonization of resistant microorganisms during drug administration (16). Third generation cephalosporins have shown good activity against Gram-negative aerobic and anaerobic rods and imipenem, a broad-spectrum beta-lactam antibiotic of the carbapenem class has shown activity against the aerobic and anaerobic flora (17).

Therapeutic doses of clindamycin may produce profound changes in the flora such as proliferation of resistant enterococci, decrease in the number of anaerobic cocci and rods, overgrowth of ***Clostridium difficile***, and decrease in the number of ***Escherichia coli*** (8, 17, **18).**

Erythromycin, at therapeutic doses, has been shown to produce a drastic reduction in the number of enterococci and streptococci. This drug has also been shown to allow colonization of the colon by anaerobic bacteria and yeasts **(8).** Tetracyclines, e.g., doxycycline at therapeutic doses, have also been shown to increase the number of resistant anaerobic bacteria (17).

B. Residue Levels of Antimicrobial Drugs and Their Effect on the Intestinal Flora

The effect of antibiotic residues in food on the intestinal flora of the consumer has been a concern for many years. However, the residue dose of antimicrobial drugs that adversely disturb the intestinal flora has not been defined. Some research has been performed for evaluating the effect of residue levels of antibiotics on different endpoints of the human intestinal flora.

Oxytetracycline at doses of 10-50 mg/day has been shown to increase the excretion of resistant coliforms in some volunteers (19, 20); and even 2 mg/day given for 7 days produced a significant increase in the proportion of resistant ***Enterobacteriaceae*** in 6 volunteers (19,21). Ampicillin given to 5 volunteers for 21 days at a dose of 1.5 mg/day produced a significant increase in resistant *E. coli* in two individuals. However, some authors have concluded that this trial lacked statistical power because there were not enough volunteers in the group (19, 22).

In vitro and ***in vivo*** model systems also have been used to study effects of antibiotic residues on human intestinal flora. Milk safe residue levels of ampicillin, oxytetracycline, dihydrostreptomycin, neomycin, sulfamethazine, and erythromycin, as determined by FDA, showed a strong potential for selecting antibiotic-resistant ***Staphylococcus aureus*** (based on MIC determinations) when the microorganism was exposed for 14 days to each drug or to combinations of three drugs (23).

Sub-inhibitory concentrations of streptomycin, nalidixic acid, rifampicin, gentamicin, chloramphenicol, tetracycline, and ampicillin were tested using a continuous flow culture system mimicking the colonic environment. A mix of three strains **of** *E. **coli*** (with and without R-plasmid and F'lac plasmid) with similar MICs for the antibiotics in study was added to the system. Tetracycline at 0.25 μg/mL (1/10 of the MIC of the susceptible strain) favored the growth of the resistant strain; however, no R-plasmid transfer was observed. Similar results were seen with chloramphenicol and gentamicin (24).

In FDA studies conducted under a research contract, a continuous flow one chambered culture system inoculated with **human** intestinal flora was used to study the effect of low doses of tetracycline, neomycin, erythromycin, and ciprofloxacin on bacterial populations, disruption of the barrier effect, metabolic activity, and development of resistant strains. Under the conditions tested, ciprofloxacin at dose levels of 0.43, 4.3, and 43 μg/mL produced a dose-dependent decrease in *E. **coli*** population. **A** decrease in the population of ***Bacteroides*** was seen with 43 and 4.3 μg of ciprofloxacin per mL of culture medium, and a decrease in susceptibility of ***Bacteroides*** to 4 μg/mL of ciprofloxacin was found in the 0.43 μg/mL culture system. Disruption of the barrier effect was evidenced by colonization of the 4.3 and 5 μg of ciprofloxacin per mL culture systems challenged with a strain of ***Salmonella kedogou*** (25, 26).

In the FDA studies, tetracycline produced a transitory dose-dependent increase in resistant *E. **coli*** strains at dose levels of 0.15, 1.5 and 15 μg/mL. Neomycin changed the proportion of short chain fatty acids at 1.78, 17.8, and 178 μg/mL, produced a dose-dependent decrease in the metabolism of bile acids, a dose-dependent decrease in azoreductase activity, and a significant increase in the percentage of resistant enterococci at 17.8 and 178 μg/mL. Erythromycin showed a dose-dependent transitory decrease in bile acid metabolism similar to neomycin, at 1.5, 15, and 150 μg/mL (27).

Sarafloxacin tested in an ***in vitro*** model simulating the colonic conditions and in broth culture inhibited the growth of *E. **coli*** in a dose-dependent manner at 0.24 and 3.7 μg/mL. The drug was less inhibitory in the model than in broth culture. The authors concluded that ***Bacteroides*** and ***Bijidobacterium*** were rather insensitive to the drug in this model (28).

The effect of low levels of antibiotics has also been studied in human flora-associated rodents. Tilmicosin at 400 μg/kg/day produced a transient increase in the number and proportion **of** enterobacteria and spiramycin at 500 μg/kg/day showed a significant increase in the number of spiramycin-resistant enterobacteria when given to **human** flora-

associated rats for 5 days (29). Low doses of ampicillin, colistin, flumequin, gentamicin, tetracycline, or streptomycin given orally for 2 weeks to gem-free mice colonized by two isogenic strains of *E. **coli,*** (one carrying an R-plasmid) showed a strong correlation between antimicrobial dose and selection of resistant ***E. coli*** strains (30). Human flora-associated mice continuously administered low doses of ampicillin (0.5 μg/mL), chlortetracycline(0.5 μg/mL), or streptomycin in the drinking water showed an increase in the number of resistant *E. **coli*** (22).

Under the FDA research contracts, the effects of residue levels of tetracycline, neomycin and ciprofloxacin were also studied in human flora-associated mice. Tetracycline at 1, 10 and 100 ppm in the drinking water for **8** weeks produced a significant increase in resistant enterococci and ***Bacteroides fragilis*** at all dose levels and ***Enterobacteriaceae*** at 10 and 100 ppm. The effect disappeared after cessation of treatment. The barrier effect to a challenge strain of ***Salmonella scharzendrung*** was also impaired at 100 ppm. Neomycin at 0.2, 2, and 20 ppm in the water did not produce any significant effect on the endpoints evaluated (counts of susceptible and resistant target bacteria, metabolic activity parameters and colonization resistance properties). Ciprofloxacin at 1, 10, and 100 ppm produced a significant decrease in total aerobes and enterococci populations. Enterobacteria decreased in a dose-dependent manner with total elimination at 10 and 100 ppm of the drug. The percentage of resistant enterococci and resistant clostridia increased during treatment with 100 ppm of ciprofloxacin. Resistant ***Bacteroidesfiagilis*** increased at 10 and 100 ppm. The barrier effect against a strain of ***Salmonella typhimurium*** was disrupted with 100 ppm of the drug, but the effects were not clear with 10 and lppm (31, 32, 33, 34, 35, 36).

C. Endpoints of Public Health Concern

The main adverse effects of antimicrobial drugs on the human intestinal flora are 1) disruption of the colonization barrier (or barrier effect) with overgrowth of potentially pathogenic microorganisms, 2) alterations of the metabolic activity of the intestinal flora, **3)** development of resistant strains, and 4) changes in bacterial populations.

Colonization barrier or effl

The colonization barrier (or barrier effect) is the property of the flora that prevents overgrowth of transient potentially pathogenic microorganisms, the outgrowth of indigenous potentially pathogenic microorganisms, and/or proliferation of antibiotic-resistant strains. The barrier effect may be disrupted by the action of any antimicrobial drug on the intestinal flora. This property is associated mainly with the indigenous anaerobic bacteria (37). **A** classic example of disturbance of the intestinal flora is that caused by clindamycin. Clindamycin is an antibiotic with activity against Gram-positive cocci and many anaerobic bacteria such as ***Bacteroidesfiagilis, Fusobacterium, Peptostreptococcus, Peptopcoccus,*** and ***Clostridium perfringens***. Diarrhea (at different degrees) associated with clindamycin treatment has a frequency of 2-20% **(38).** **A** severe syndrome, pseudomembranous colitis, caused by a ***Clostridium difficile*** toxin has been described in up to 10% of the patients treated with this antibiotic. The syndrome may be

fatal if not treated ***(38)***. Although ***Clostridium difficile*** colitis was initially associated with clindamycin, ampicillin and cephalosporins have also been cited as common causes of this syndrome (39).

Overgrowth of transient pathogenic microorganisms or commensal organisms of the intestinal flora, due to disruption of the barrier effect, is an effect that was linked to antibacterial drugs many years ago. This overgrowth may result in enteric infections such as staphylococcal enterocolitis, infections due to ***Salmonella, Klebsiella,*** *E.* ***coli, Pseudomonas, Proteus, Yersinia enterocolitica,*** and others. Studies performed in volunteers have shown that amoxicillin, cefotaxime, clindamycin, or co-trimoxazole disrupt the barrier effect and facilitate colonization of challenge strains of *K.* ***pneumonia*** and *E.* ***cloacae.*** An increase in the number of resistant Gram-negative bacteria, enterococci, and yeasts was also seen in the feces of the volunteers (40, 41, 42, 43). Disruption of the barrier effect resulting in increased populations of normal flora components such as enterococci and ***Clostridium perfringens*** may also produce antibiotic-induced diarrhea (44).

Selection of resistant straihs

Low or subtherapeutic doses of antimicrobials can increase the percentage of resistant microorganisms in the normal intestinal flora. Studies have shown that healthy people can harbor a large number of antibiotic resistant bacteria in the intestinal flora, mainly of the ***Enterobacteriaceae*** family (almost 40% of people continuously carry resistant bacteria, although the numbers and types vary daily) ***(6)***. Drug-resistant commensals bearing R-plasmids could also increase in number due to low doses of antimicrobial drugs favoring plasmid transfer to pathogenic bacteria and the addition of new genes on a pre-existing R-plasmid. ***Escherichia coli,*** a normal resident of the intestinal flora, may be resistant to multiple antimicrobial drugs and may be important reservoirs of resistant plasmids from which genes coding for resistant determinants may be transferred to human pathogens (45, 46).

There are different forms of resistance selection in the intestinal flora. One is the selection of species that are intrinsically less susceptible than others, such as ***Clostridium difficile,*** yeasts, various Gram-negative species, and Enterococcus. Another form is the selection of mutants from normally susceptible strains that exhibit decreased susceptibility to antimicrobials. These mutations may occur in genes that regulate different functions of the bacterial cell such as outer membrane proteins involved in the diffusion of antibiotics across the bacterial cell wall or the regulation and expression of beta-lactamases. Multiple antibiotic resistant phenotypes may arise by movement of mobile genetic elements such as plasmids or transposons that can carry resistance genes. This transposition may occur in the absence of antibiotic selective pressure, but antibiotics have been shown to increase transposition. For example, the transmission of tetracycline resistance in Gram-positive bacteria by a conjugated transposon is stimulated by sub-inhibitory concentrations **of** tetracycline (47).

The cost of resistance can be measured in terms of increased morbidity (extended length of therapy or hospitalization), mortality (death due to infections with antibiotic resistant strains), and cost of therapy. Unrecognized costs associated with antibiotic resistance include persistence of resistance in a population or in a patient. Resistance may or may not decrease when the use of an antibiotic is suspended (47).

Alteration of the metabolic activity of the intestinal flora

The intestinal flora catalyzes a number of reactions including hydrolysis, reductions, degradations, and synthesis. The biotransformation of compounds may be beneficial or have adverse toxicological consequences for the host.

Antimicrobial drugs may alter the ecological balance of the flora resulting in alteration of its biotransformation capacity to change the original activity or toxicity of compounds (5, 6). Indicators of the metabolic activity of the intestinal flora include measurement of hydrolytic enzymes (beta-glucosidades, beta-glucuronidase), reductases (nitroreductase, azoreductase, nitrate reductase), metabolism of bile acids and cholesterol, production of short chain fatty acids, determination of cellular fatty acids, sulfate reduction, and others. The assessment of these activities is indirectly related to the barrier effect, although the observed changes cannot always be related to public health concerns (5, 45, 48).

The effect of changes in the metabolic activity of the flora will depend on the specific activity and the physiological or pathological conditions with which it has been associated. For example, evaluation of bile acids metabolism by the intestinal flora may indicate drastic changes in flora composition because primary bile acids are metabolized by the flora to secondary bile acids and excreted or passively absorbed and re-circulated. The ratio of primary to secondary bile acids may indicate profound changes in bacterial composition. Secondary bile acids metabolites have been shown to be cancer promoters, therefore, an increase in the ratio of secondary bile acid metabolites may increase the risk of colon cancer for the susceptible individuals. In addition, bile acids have been associated with an increase in serum cholesterol (49).

Cellular fatty acids also indirectly relate to changes in the proportion of colonic species. Short chain fatty acids, present in high concentrations in the colon and in feces, are end-products of flora metabolism. Changes in the molar ratios of short chain fatty acids due to the effect of antimicrobial drugs may also indicate changes in bacterial populations (6, 50). Drugs such as ampicillin, clindamycin, vancomycin and bacitracin reduce the levels of fecal short chain fatty acids (49). Therapeutic doses of bacitracin and vancomycin have been shown to drastically reduce fecal short chain fatty acid excretion while doxycycline, nalidixic acid, ofloxacin, and co-trimoxazol have little or no effect. Erythromycin has a moderate effect on fecal short chain fatty acids excretion. Other antimicrobial drugs can elevate the levels of a specific fatty acid (50). The reduction of cholesterol to coprostanol by intestinal bacteria is another way to monitor the stability of the intestinal flora. However, human populations vary considerably in their degree of cholesterol reduction (49).

The level of enzymes in feces represents potential bacterial activity of the microflora. Conjugated compounds and complex polysaccharides are metabolized in the colon by bacterial glycosidases (beta-glucuronidase being the most important one). The beta-glucuronidase activity in the colon is mainly the responsibility of the ***Bacteroides*** species. Changes in its activity may result in changes in the capacity of the flora to influence the pharmacokinetics of drugs, genotoxins, tumor promoters, and other bioactive compounds. Another hydrolytic enzyme, beta-glucosidase, hydrolizes a broad range of sugar conjugates. Several bacteria produce this enzyme, such as the streptococci and lactobacilli. However, since these species are not present in high numbers in the colon, the majority of the beta-glucosidase activity is again the responsibility of the ***Bacteroides*** group. Of the reductase enzymes, azoreductase activity is also responsible for reduction of azo dyes (49).

Changes in bacterial populations

Drastic changes in bacterial populations by antimicrobial drugs may disrupt the colonization resistance properties of the intestinal flora, the metabolism of compounds that undergo enterohepatic circulation (estrogens, vitamins, cholesterol, protoporphyrin, and bile acids), or the metabolism of drugs undergoing enterohepatic circulation resulting in increasing blood levels of the drugs (6, 45). For example, therapeutic doses of tetracycline and erythromycin reduce the population of ***Eubacterium*** in the colon, which is responsible for the reduction of digoxin, a cardioglycoside drug. Dangerous blood levels of digoxin may be reached in patients treated with these antibiotics (6). Antimicrobial drugs can also influence estrogen metabolism by eliminating intestinal bacteria responsible for their deconjugation and reabsorption of the free hormone. The result is an increase in the fecal excretion of conjugated estrogens. In addition, the contraceptive effect of synthetic steroids may be diminished by the effect of antibiotics due to changes in the intestinal flora that result in alteration of the metabolism of the chemicals and a decrease in the circulating half-life of the estrogen dose (6).

D. Model Systems for Evaluating Endpoints of Concern

In vitro and ***in vivo*** tests and model systems have been used to study the effects of antimicrobial drugs on the human intestinal flora. These models attempt to simulate the human colon and its microbial population.

MIC data

Quantitative ***in vitro*** antimicrobial susceptibility testing on bacteria from the colonic flora has been used by pharmaceutical sponsors and presented to international organizations such as **JECFA** and the CVMP for the assessment of the human food safety of veterinary antimicrobial drug residues. These data, presented as **MICs,** have been incorporated into formulas for establishing the ADI for antimicrobial veterinary drug residues in food. The advantages of determining **MICs** are simplicity, rapidity, **and** low cost. However, use of this testing for assessing the human food safety **of** veterinary antimicrobial drug residues in the food from treated animals has disadvantages. Some of the disadvantages of MIC

determinations for this purpose are the following: (1) because they are done on pure bacterial cultures, they are not representative of the ecological system in the human intestinal tract; (2) they do not take into account the representativeness of the bacteria studied, the pH, anaerobic conditions of the colon, or *in vivo* conditions such as absorption, metabolism, enterohepatic circulation, and fecal concentration of the drug; (3) they do not assess long-term effects of antimicrobials on the intestinal flora; (4) they do not allow quantitation of minor populations of resistant bacteria (not enough selected clones); (5) they do not assess perturbations of the intestinal flora such as disruption of the barrier effect and changes in enzyme function (51, 52, 53, 54).

In vitro model systems

Static batch, semi-continuous, and continuous flow culture systems mimicking the colonic environment have been developed for studying the effects of diets, food additives, and drugs on the intestinal flora. Static (batch) cultures are useful for performing short-term metabolism studies and for determining the potential of a drug of being inactivated due to binding or chemical transformation. However, the bacterial composition changes with time. Semi-continuous and continuous culture systems include test systems inoculated with one or more types of bacteria or with feces to which fresh medium is added and used; culture media is removed periodically or continuously, depending on the model. Different models have been developed, ranging from a single vessel to a two or three-stage model. The advantage of these models is that they model the intestinal flora and allow the study of long-term exposure to different drug concentrations. These systems can be used for determining NOELs for microbiological endpoints (functional endpoints, resistance emergence, and barrier effect). However these models do not take into account host metabolism, the bacterial populations are still lower than those in the colon, and expertise is required to set up and maintain the systems. Studies using a semi-continuous culture system have also shown high variability for determining the NOELs in colonization resistance studies using human intestinal flora. These variations could be due to fecal inoculum differences or other factors (48, 54, 55).

Simulated gut models mimicking the passage of food through the human gastrointestinal tract have been developed. In such models, the test substance is incubated sequentially under conditions similar to the stomach and the intestine, bacteria are added to the medium, and survival is determined by microbiological plate counts. This model is relatively inexpensive and simple to perform; however, it does not resemble the complexity of the intestinal flora and does not account for host metabolism. The endpoint to be studied is survival of indicator bacteria from the gut (54).

A gastrointestinal simulation model was also developed for determining NOELs and establishing ADIs for antimicrobial drug residues based on MIC values for indicator bacteria. The indicator bacteria are checked for changes in MIC due to exposure to low concentrations of antimicrobial drugs (56). A similar model was used to study the effect of sarafloxacin on *E. coli*, *Bacteroides fragilis*, and *Bidifobacteria* strains (57). Another similar model was developed for studying the effect of antibiotic drug residues on

intestinal flora under anaerobic conditions. The model is inexpensive, easy to set up and studies drug exposure in an intermittent manner providing some insight on the interaction between the colonic conditions, residue levels of drugs and the resultant antimicrobial activity (58). Although this model takes into account some aspects of host metabolism in the colon that are not considered in the standard MIC test, the model has all the disadvantages of pure culture testing described above under "MIC data" (54).

A semi-continuous culture system was developed for maintaining the human colonic flora and studying their interaction and fermentation processes for a long period of time (81 days). The authors concluded that the model could be suitable for studying microbial activities and bacterial populations of the colon (59). A semi-continuous flow culture system inoculated with human intestinal flora was used to study the activity of the flora on the metabolism of three different chemical classes of xenobiotics *(60)*. The same model was also used to determine if the metabolic activity of the flora could be maintained *in vitro.* The model proved to be useful for maintaining the diverse population of the colon and the metabolic activity of the flora for prolonged periods of time (61). The semi-continuous model has also been studied as a possible model for determining NOELs for antimicrobial drug residues based on the disruption of the barrier effect of the intestinal flora. The model was capable of detecting a dose-response effect to clindamycin. However, the responses varied among experimental runs and it was concluded that further studies were needed to investigate the causes of variability before determining the usefulness of this model for assessing barrier effect for regulatory purposes *(55)*.

A continuous flow culture system has been used to study interactions between representative strains of the human colon and strains of seven enteropathogenic bacteria (62). The same model, inoculated with feces from human volunteers, was used for studying the function of the colonic bacteria through time and proved to be able to maintain actively fermenting viable cultures for at least 21 days **(63).** A three-stage continuous culture system inoculated with mixed populations of human intestinal bacteria was developed to study the effect of mucin on dissimilatory sulfate reduction and methanogenesis. Each stage represented a section of the large intestine with specific nutritional and pH characteristics. The model was able to sustain bacterial growth for 120 days and it was appropriate for these types of studies **(64).**

The effect of subinhibitory concentrations of streptomycin sulfate, nalidixic acid, rifampicin, gentamicin, chloramphenicol, tetracycline, and ampicillin on the development of resistant *E. coli* strains was studied utilizing a continuous flow culture system *(65).* The **FDA** funded research also studied the applicability of a continuous flow one-chambered culture system inoculated with feces from healthy volunteers for determining the effects of low doses of tetracycline, neomycin, erythromycin, and ciprofloxacin on various mirobiological endpoints of the human intestinal flora (see section B above). Data from preliminary studies performed to establish the culture system and develop the assays for the different endpoints are described in TechLab reports (66, 67, 68, 69, 70, 71). The detailed results of this research can be found in the reports of the **FDA** contract for each drug (25, 26, 27, 72, 73, 74, 75, 76).

In vivo model systems

Human volunteers, beagle dogs, pigs, conventional rodents, gnotobiotic rodents, and human flora-associated rodents have been used to study the effect of substances on the intestinal flora. The human model is the most appropriate model. However, while the human model is very useful for clinical studies, toxicological studies are not possible because there are ethical considerations and the number of volunteers is a limiting factor for the power of the studies. The advantage of using conventional animals is that the flora can be monitored at different sections of the intestinal tract, dietary environmental factors can be controlled, and many microbiological endpoints can be evaluated. However, extrapolating the findings to the human is problematic because of differences in bacteria composition and metabolism. Gnotobiotic animals are useful for studying host-bacteria or bacteria-bacteria interactions. However, their relevance to the normal human ecosystem is questionable. Rodents (rats or mice) inoculated with diluted human feces are suitable for performing studies on human flora interactions and metabolism because the implanted flora retains many of the characteristics of the human flora. These models are easy to control, a large number of subjects can be used to obtain statistical power for the studies, and metabolic and ecological interactions of the flora, as well as colonization resistance to challenge bacteria, can be studied. However, they are expensive, difficult to set up and maintain and the gut physiology of the animal may not be similar to that of humans (48, 53, 54).

Studies on the effect of low doses of antimicrobials on human volunteers have shown that oxytetracycline produced a transient effect on the emergence of resistant coliform bacteria (20) and also increased the number of resistant ***Enterobacteriaceae*** in the fecal flora of adult volunteers (21). Ampicillin and streptomycin given orally to human volunteers at a dose of 15 mg/day of each drug for 21 days significantly raised the concentration in the feces of *E. coli* resistant to ampicillin, chlortetracycline and streptomycin. The dose of 1.5 **mg** ampicillin gave less significant results; the increase in the population of resistant *E. **coli*** occurred in only two volunteers (22).

Beagle dogs have been used to study the effect of low levels of oxytetracycline, dihydrostreptomycin, virginiamycin, and penicillin on the emergence of resistant strains in the intestinal microflora. Oxytetracycline at 10 μg/g diet for **44** days produced a **shift** from a predominantly drug-susceptible population of enteric lactose-fermenting bacteria to a multiple antibiotic resistant population. No shift was observed with 2 μg/g (77). Dihydrostreptomycin at 2 and 10 μg/g diet produced shift fi-om a streptomycin-susceptible to a streptomycin resistant coliform fecal population (78). Virginiamycin at 55 μg/g diet resulted in an increase in the proportion of lactose-fermenting bacteria in the feces that were resistant to ampicillin, dihydrostreptomycin, tetracycline, or chloramphenicol. Penicillin at 110 μg/g diet had a similar effect (79). The power of the tests was limited because of the inter-animal and day-to-day variability of the flora.

Germ-free rodents inoculated with specific bacterial strains of the human intestinal flora *(E. coli* strains with and without a tetracycline-resistant plasmid) have been used to study

bacteria-bacteria interactions due to minimal doses of antimicrobial drugs. The results showed that *in vivo* interactions between the three strains were very different from those obtained *in vitro,* with the tetracycline-resistant strain becoming dominant within one day and replacing the sensitive *E. coli. In vitro* studies had shown that the *E. coli* resistant strains were repressed by the sensitive strains. It is concluded that *in vivo* studies should be performed for the determination of no-effect levels of antibiotic residues in the gut (80). This model has also been used to study the effect of minimum doses of antibiotics ampicillin, colistin, flumequin, gentamicin, tetracycline, and streptomycin) on the selection of a resistant plasmid in germ-free mice colonized with two isogenic strains of *E. coli* (one carrying a resistant plasmid). The antibiotics were given to mice in the drinking water at dose levels ranging from 0.9 to 12.8 μg/mL. Ampicillin, gentamicin, tetracycline, and streptomycin increased the number of resistant bacteria. The minimum selecting level for resistance was defined as the drug concentration producing a 10-fold increase in the resistant population compared with that of the inoculum and to a 100-fold increase compared with the control group (30). This model does not represent the intestinal ecosystem in which many types of microorganisms coexist **and** interact with each other.

Pig flora-associated mice (mice implanted with fecal flora from pigs) have been used to study the effect of low doses of bambermycin *(5* μg/mL of drinking water), carbadox (50 μg/mL), chlortetracycline (20 μg/mL, and olaquindox (50 μg/mL) on the emergence of resistant coliforms. The occurrence of drug-resistant coliforms was higher in mice given chlortetracycline and lower in mice dosed with bambermycins; olaquindox and carbadox did not change the,proportion of resistant coliforms in mice feces **(8** 1). The model was recommended for studying the development of resistant strains due to low doses of antimicrobials using other animals' flora or human intestinal flora.

Germ-free rodents colonized with human intestinal flora is the *in vivo* model most studied to date because it incorporates the gross bacterial composition of the flora, the barrier effect, and other functions of the flora in the human. The human flora-associated mice **(HFA** mice) model has been used extensively to study the effects of antimicrobials on flora composition and on resistance to pathogen challenge (45).

Germ-free mice inoculated with human flora and with fecal anaerobes were used to study the gross composition of the flora before and after implantation in the mice and the barrier effect of the anaerobes against *Pseudomonas aeruginosa* in immunocompromised mice. The gross composition of the flora before and after implantation was similar and remained stable after five weeks. In addition, the implanted flora and the anaerobes induced an antagonistic effect against *E. coli.* In contrast to the complete flora, the anaerobes were not invasive in immunosuppressed mice and induced colonization resistance and antagonism against *Pseudomonas aeruginosa* **(82).** The same model has been used to study the effect of erythromycin (dose levels of > 1,000 μg/g in the human donor and in mice) on the barrier effect of the human flora. The drug did not reduce colonization resistance to *Candida albicans, Clostridium perfringens*, and erythromycin-sensitive *E. coli;* however, it reduced at some degree colonization resistance against *Pseudomonas aeruginosa, Clostridium difficile* and erythromycin-resistant *E. coli* (83).

The effect of high doses of nifurzide and nifuroxazide on bacterial populations and on the colonization resistance to enterotoxigenic *E. coli* and *Shigella flexeri* was also studied in human flora-associated mice. Nifurzide significantly reduced colonization resistance to *E. coli* and Shigella flexeri **(84).**

The effect of norfloxacin on the colonization resistance properties of the human intestinal flora was studied in in HFA-mice. Resistance to colonization by exogenous bacteria was reduced for 2/14 of the strains tested *(Pseudomonas aeruginosa* and *Candida albicans)* (85).

More recently, the HFA-mice model was evaluated as a model for studying persistence of the human flora in the gnotobiotic mice, the metabolic activity of the flora, and the colonization resistance to a ***Salmonella*** *typhimurium* strain. The model seemed appropriate for studying colonization resistance properties of the flora, since a *Salmonella typhimurium* strain could not be established in the HFA-mice but did invade the intestine of a germ-free mice **(86).**

HFA-rats have also been studied to evaluate the potential effect of low doses of tilmicosin and spiramycin on bacterial composition and on the development of resistant strains. Low levels of tilmicosin and spiramycin given orally to HFA-rats for 5 days showed no major changes in the anaerobe population but the number of spiramycin resistant enterobacteria increased significantly fi-om day 2 (29). This model had been used earlier to study the formation of apparent total N-nitroso compounds in the human intestinal flora of implanted rats **(87).**

The FDA funded research studied the effect of low doses of tetracycline, neomycin and ciprofloxacin on the human intestinal flora using the HFA-mice model. The microbiological endpoints evaluated in the studies were similar to those evaluated in the *in vitro* studies discussed in B above. Detailed results of these studies are found in the quarterly reports to the FDA **(3** I **,32, 33, 34, 35, 36, 88, 89).**

E. International Approaches for the Regulation of Antimicrobial Drug Residues in Food

The safety of antimicrobial residues in food have been assessed internationally by three organizations: **1)** the Codex Alimentarius Commission (CAC); 2) the European Medicines Evaluation Agency (EMEA); and **3)** International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH). Each organization has scientific groups that provide advice on the safety of veterinary drug residues and appropriate studies to determine their safety. The scientific advisory groups make recommendations that will later become standards when approved by the organizations. The CAC sets standards for veterinary **drug** residues based on recommendations made by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) through the Codex Committee on Residues of Veterinary Drugs in Food. The EMEA sets standards based on recommendations from the Committee for Veterinary Medicinal Products (CVMP). The VICH recommends requirements and protocols for determining **human** food safety of veterinary drugs based on recommendations from the Safety Working Group (SWG).

Joint Expert Committee on Food Additives (JECFA)

JECFA initially addressed the microbiological safety of veterinary drug residues in foods in June of 1987. The Committee concluded that the antimicrobial properties of veterinary drug residues would become the determining factor in safety evaluation when the toxicity of the substance is so low that their residues could be tolerated without any withdrawal period. In such a case, the safety of the residues would be based on their danger to human health due to their selective pressure on the intestinal flora favoring growth of microorganisms with natural or acquired resistance (90).

In 1990 the Committee concluded that the most important characteristics of the intestinal flora to be considered when assessing the microbiological risk of antibiotic residues in food are the proportion of anaerobic bacteria, the stability of the flora, and the barrier effect. Thus, the Committee determined that the safety evaluation of antimicrobial residues should be based on data related to bacteria that constitutes the flora, taking into account the barrier effect. If human data are not available, animal studies might be considered. The Committee encouraged the validation of animal models such as haloxenic rodents implanted with human intestinal flora and also concluded that, in the absence of *in vivo* data, *in vitro* data such as MIC's could be used on a temporary basis for safety evaluations (91).

In 1991, JECFA for the first time calculated the ADI for an antimicrobial drug (spiramycin) using MIC data from four species of the dominant anaerobic flora. A formula was developed using the modal MIC of the bacteria tested, safety factors to cover to different variables, the daily fecal bolus, the fraction of oral dose available, and the weight of humans (92).

In 1994, JECFA concluded that the evidence of risk due to low levels of antimicrobial residues is minimal and other methods for studying the microbiological endpoints may be useful for assessing this risk. MIC data would continue to be accepted for determining ADIs until other methods could be developed and accepted for this purpose (93).

In 1995, JECFA discussed a new 'decision tree' approach to the safety evaluation of antimicrobial residues (94), and in 1996 the Committee concluded that more research was needed concerning the public health risk of antimicrobial residues and their effects on the human intestinal flora. They recommended that MIC data should not be the only method used to calculate an ADI and that data from *in vitro* or *in vivo* model systems or any other relevant data should be used for setting ADIs. In absence of human data, data from *in vivo* model systems (e.g., human flora-associated rodents) or *in vitro* models (e.g., continuous flow cultures) could be used for determining ADIs for antimicrobial drugs. They recognized the limitations of the formula method, and the formula using MIC data was again modified. The Committee recommended the development and validation of *in vitro* and *in vivo* model systems that would be more appropriate for determining NOELs and setting ADIs for antimicrobial residues. The Committee also concluded that, when sufficient data are available, no additional microbiological information on drug effects on

the human intestinal flora would be necessary if the residues in food do not exceed 1.5 mg/person/day (95).

In 1998, the Committee determined the ADI of several antimicrobial agents (gentamicin, sarafloxacin, tetracyclines) based on effects on ***in vitro*** studies and using the formula approved in their 47th meeting. The tetracyclines' ADI was determined based on the development of resistant *E. coli* seen in a human study and also confirmed in a study conducted using a continuous flow culture system (96). In February 2000, the Committee determined the ADI for lincomycin using the 'decision tree' approach discussed in 1995.

Committee for Veterinary Medicinal Products (CVMP)

The CVMP began evaluating the human food safety of veterinary drug residues in 1995. Their primary focus on microbiological **risk** is the assessment of effects **and** safety of antimicrobial drug residues on the human intestinal flora (97).

Initially, the CVMP applied the approach followed by JECFA in 1992 for the evaluation of antimicrobial drug residues in food. However, some difficulties were encountered and in March 1994, the CVMP adopted a guideline that would be used for the next five years until further review of the approach. Three evaluation approaches are accepted by the CVMP: human data with an appropriate safety factor; data to demonstrate the no-observed effect level (NOEL) obtained in (HFA) rodents when the induction of resistance and reduction of the barrier effect are studied; or the calculation of a microbiological ADI from ***in vitro*** MIC data obtained under conditions similar to those in the colon. The CVMP developed a formula slightly different than the JECFA formula.

In April of 2001, the CVMP published for consultation a revised guideline entitled "Revised Guideline on Safety Evaluation of Antimicrobial Substances Regarding the Effects on Human Gut Flora". The revised guideline states that the current CVMP microbiological ADI formula will continue to be used **as an** interim measure until the adoption of a harmonized VICH guideline. The approaches to calculate the ADI remain the same; however, the formula including MIC data was slightly modified. The revised guideline states that the two endpoints of concern that should be addressed in the determination of a microbiological ADI are reduction or elimination of the barrier effect of the normal flora and development of and/or increase in the pool of antibiotic-resistant strains of potentially pathogenic microorganisms.

The CVMP calculates both a toxicological and a microbiological ADI for antimicrobial drugs. The most relevant ADI (usually the lowest) is used to determine the maximum residue limit (MRL) **(98).**

International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH)

The VICH, a trilateral program with representatives from the European Union, the United States, Japan, and attendance from Australia/New Zealand, initially addressed the safety of antimicrobial drug residues in April of 1999. At that time, the VICH SWG agreed to charge a sub-group of experts with attending the 1999 **FDA** workshop "Microbiological Safety of Drug Residues in Food". The group should also write recommendations to the SWG for the regulation of antimicrobial residues based on effects on the human intestinal flora. The expert group recommended that a Task Force be formed with microbiology experts in human intestinal flora ecology. The Task Force would review all information available and make recommendations to the SWG on testing methods and protocols for determining NOELs for antimicrobial drug residues based on effects on human intestinal flora.

The Microbial Safety Task Force met four times (July of 2000, May of 2001, December of 2001, and April of 2002) and completed the SWG's mandate to recommend testing methods **and** protocols for the safety evaluation of antimicrobial drug residues in food. The recommendations are currently being reviewed and considered for adoption by the SWG, and will be published as a VICH guideline.

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